

Molecular subtyping of feline immunodeficiency virus from domestic cats in Australia

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Objective To determine the prevalent subtypes of feline immunodeficiency virus (FIV) present in the domestic cat population of Australia.

Method Blood samples were collected from 41 FIV antibody positive cats from four cities across Australia. Following DNA extraction, polymerase chain reaction (PCR) was performed to amplify the variable V3-V5 region of the envelope (*env*) gene. Genotypes were assessed by direct sequencing of PCR products and comparison with previously reported FIV sequences. Phylogenetic analysis allowed classification of the Australian sequences into the appropriate subtype.

Results Of the 41 FIV samples, 40 were found to cluster with previously reported subtype A isolates, whilst the remaining sample grouped within subtype B.

Conclusions Subtype A was found to be the predominant FIV subtype present in Australia, although subtype B was also found. These results broaden our knowledge of the genetic diversity of FIV and the associated implications for preventative, diagnostic and therapeutic approaches.

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FIV	Feline immunodeficiency virus
LTR	Long terminal repeat
PCR	Polymerase chain reaction
<i>env</i>	Envelope gene
PBS	Phosphate buffered saline
HIV	Human immunodeficiency virus

Feline immunodeficiency virus (FIV) is an important pathogen of domestic cats throughout the world. This virus was first identified in a group of cats in a Californian cattery that were showing signs consistent with an immunodeficiency syndrome.¹ FIV is a T-lymphotropic virus that establishes persistent infection in the domestic cat causing a gradual depletion of CD4⁺ lymphocytes and ultimately immunosuppression.^{2,3} Clinical disease is generally seen after a variably long latent period and consists of secondary or opportunistic infections, haematological abnormalities and occasionally neoplasia and neurological disease.²⁻⁴

FIV is a member of the *Retroviridae* family, genus *Lentivirus*. The genome is comprised of three main structural genes, termed *gag*, *pol* and *env*, which encode the internal structural proteins, viral enzymes and envelope glycoproteins respectively.⁵ The *env* region of the genome has an important role in the pathogenesis of the virus. It is involved in the interaction of the virus with susceptible host cells and contains immunodominant neutralisation domains and determinants for cell tropism.^{2,6}

The viral *env* gene shows significant sequence diversity in comparison to the relatively conserved *gag* and *pol* regions. Nine distinct variable regions (V1-V9) have been identified within the *env* gene.⁶ Based on sequence differences in the variable V3-V5 region of the *env* gene, FIV isolates have been divided into five phylogenetic subtypes designated A, B, C, D and E.^{7,8} Nucleotide sequence diversity ranges from 17.8% to 38.0% between different subtypes whereas up to 15% divergence is found within a subtype.^{9,10} In general, similar phylogenetic clades, and hence similar subtype assignments, are obtained when nucleotide sequences from other genetic regions are analysed, particularly the *gag* gene.¹⁰⁻¹⁴ However, the high variation of the *env* region makes it the preferred region for subtyping.

The five phylogenetic subtypes of FIV do not exist within clear geographical boundaries. Subtypes A and B are the predominant subtypes with a relatively wide geographical distribution in comparison to subtypes C, D and E. Subtypes A and B have been found in the United States, Canada, Europe and Japan.^{7,15} Subtype C occurs predominantly in Canada, Vietnam and Taiwan.¹⁵⁻¹⁷ Subtypes D and E have a more restricted distribution and have been reported in Japan and Argentina respectively.^{12,18} The spread of subtypes is thought to be associated with people travelling and migrating with cats.⁷

While many subtypes are widespread, the prevalence of FIV around the world is variable. Australia is considered to have a reasonably high FIV seroprevalence with reports of 6.5 to 29.0% in healthy cats and 20.0% to 32.3% in sick cats.¹⁹ However, little work has been performed to determine the prevalent subtypes. Greene et al (1993) sequenced the *gag* and *pol* genes of four Australian isolates and these were found to cluster within subtype A.¹¹ Subsequent analysis of the Long terminal repeat (LTR) of three isolates gave similar results.¹⁴ Two Brisbane cats were included in a worldwide survey using an indirect analysis method, the heteroduplex mobility assay on PCR fragments of the V3-V4 region of the *env* gene. These two FIV isolates were also found to cluster within subtype A.¹⁵ These preliminary data are based on only a few samples and a more comprehensive survey of subtypes is required.

Developing an effective vaccine that is protective against the many genetic variants has been a major challenge in FIV as well as human immunodeficiency virus (HIV) research. Several vaccines, employing different conventional and molecular strategies, have been developed and trialled. The conventional vaccines containing inactivated whole virus or fixed infected cells have proven to be more successful than the molecularly derived vaccines.^{20, 21} Single subtype vaccines have provided good protection against challenge with the vaccine strain and closely related strains. However, little protection had been achieved against more divergent strains of the same subtype or other subtypes.^{20,21}

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In 2002, a dual subtype FIV vaccine became commercially available in the United States (Fel-O-Vax® FIV, Fort Dodge Animal Health) and it has subsequently become available in other regions of the world, including Australia. This vaccine contains inactivated infected cells and whole viruses from subtypes A and D. It has been found to be efficacious against challenge with homologous subtypes and also against heterologous subtype B challenge.²²⁻²⁴

Identifying the type and diversity of FIV strains in areas where vaccination is to be implemented is, therefore, important. Not only may it help establish the potential efficacy of the vaccine but it could also play a role in the development of diagnostic assays that could be used to differentiate between cats infected with FIV from those that have been vaccinated.

In this study, we investigate the genetic diversity of FIV subtypes in Australia by determining the proviral DNA sequences of the V3-V5 region of the *env* gene obtained from blood samples from cats from several areas in Australia.

Materials and methods

Proviral samples

Anticoagulated blood samples from 41 FIV-antibody positive cats were received from Brisbane (8), Melbourne (10), Perth (10) and Sydney (13). The samples were collected from cats that presented to clinics for diagnostic workup. The AGENT™ FIV Antibody test was initially used to determine whether a cat was infected with FIV.

Age, sex, breed and the presence of any clinical disease was also recorded for most cats.

Reference strain

A reference strain, T91, was used as a positive control for the PCR reactions. This isolate was first described in 1992 and originated from an adult male cat from Perth that was naturally infected with FIV. This isolate was maintained in a T-lymphoblastoid cell line (MYA-1).^{11,25}

DNA extraction

Genomic DNA was extracted from 200 µL of whole blood using the Wizard® SV Genomic DNA Purification System (Promega), according to the manufacturer's instructions. For the reference strain, T91, the tissue culture cells were washed twice with PBS before extraction. The genomic DNA was eluted in a total volume of 250 µL nuclease free water and stored in aliquots at -20°C until use.

PCR

A region encompassing the V3-V5 region of the *env* gene was amplified using the polymerase chain reaction. The majority of sequences were amplified with the primers (Hv3f-Hv5r) of Pecoraro et al (1996).¹⁸ These primers correspond to nucleotide positions 7322-7344 and 8049-8027 respectively of the published FIV TM2 sequence.²⁶

However, several samples could not be sufficiently amplified with these primers. Primers were designed that amplified the corresponding region in one or two overlapping fragments. Primers su3-su4 amplify the region between nucleotides 7314 and 7806 of the published FIV Petaluma sequence and the primer pair su5-su6 amplify from nucleotides 7660 to 7942.²⁷

Primer sequences are as follows:

su3 5' ATWCCAAAATGTGGATGGTGG 3'

su4 5' AATAAGGTCATCTACCTTCAT 3'

su5 5' AATCCTGTAGATTGTACCATG 3'

su6 5' TCCTGCTACTGGRTTATACCA 3'

PCR amplification was performed in a reaction mixture (12.5 µL) containing 2 mM (su3-su6, su3-su4, su5-su6) or 2.8 mM (Hv3f-Hv5r) MgCl₂, 200 µM of each deoxynucleoside triphosphate, 0.4-0.8 µM of each primer and one unit Expand High Fidelity Enzyme mix (Roche®). Reaction conditions were an initial denaturation at 94°C for one minute followed by 40 cycles of denaturation at 94°C for one minute, annealing at 57°C (primers su3-su4 and su5-su6) or 55°C (primers Hv3f-Hv5r) for one minute and extension at 72°C for one minute. A final extension at 72°C for 10 minutes was performed.

For some products, the band of the appropriate size was excised from an agarose gel. These fragments were gel purified followed by sodium acetate and isopropanol precipitation and used to reseed a PCR with conditions as described above, with the exception of 1.5 mM MgCl₂ and an annealing temperature of 60°C in a 30 cycle PCR.

Sequencing

PCR amplified fragments were purified using the MinElute™ PCR Purification Kit (Qiagen) and directly sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Kit (v3.1) with the PCR primers. The sequences were resolved on an Applied Biosystem automated DNA sequencer. For two samples, P9 and B8, only the first (su3-su4) and second (su5-su6) fragment respectively were successfully amplified and sequenced.

Sequence and phylogenetic analysis

Sequence analysis and alignments were performed using programs from the Australian National Genomic Information Services (ANGIS).²⁸ The amino acid sequences obtained in this study were aligned with 30 previously reported sequences using ClustalW,²⁸ to evaluate conservation of amino acid sites such as cysteine residues and N-linked glycosylation sites.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0.²⁹ A multiple sequence alignment was created with ClustalW using the same sequence data base described in Figure 1 legend. Genetic distances between pairs of sequences were determined using the Kimura 2-parameter model for nucleotide sequences and using the p distance for amino acid sequences. An unrooted neighbour joining tree was then constructed to determine the phylogenetic relationship. For comparison, trees were also created based on the criteria for minimum evolution and both nucleotide and amino acid sequence based trees were generated. In each instance, bootstrap analysis was performed on 1000 repetitions to evaluate the consistency of branching order. Information on the direction of evolutionary change cannot be deduced as there is no outgroup sequence.

Results

Cats

The mean age of the cats in this study was 10.2 years. Of the 41 cats for which sex was recorded, 39 were male. Breed was

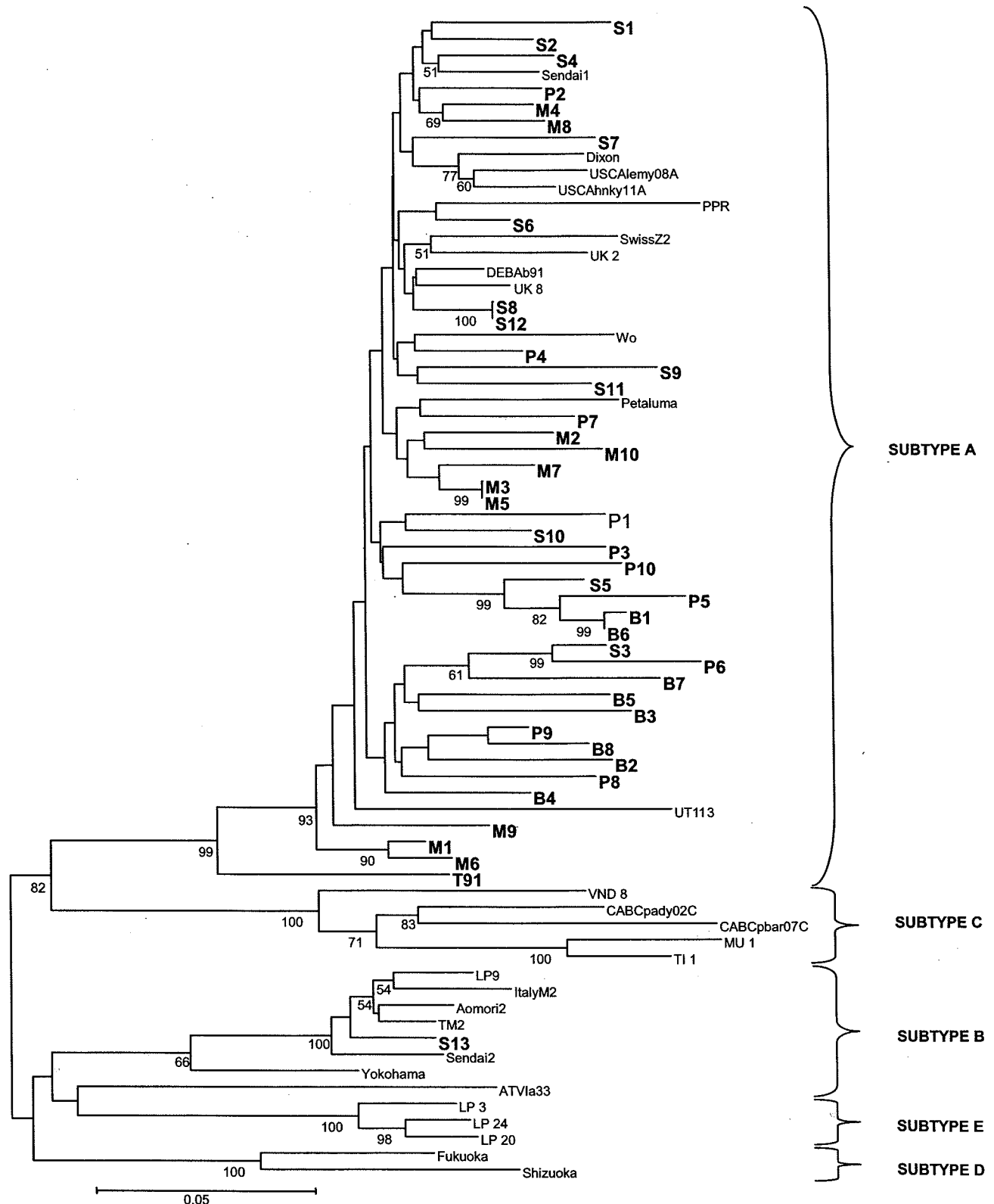


Figure 1: Unrooted neighbour joining tree inferred from the alignment of V3-V5 env nucleotide sequences of FIV.

Bootstrap values, as a percentage of 1000 bootstrap replicates, are shown on branches where values are greater than 50%. Branch lengths are drawn to scale. The subtypes A, B, C, D and E are indicated. The Australian isolates from this study are designated B for Brisbane samples, M for Melbourne samples, P for Perth samples, S for Sydney samples and T91 for the reference strain and are shown in bold. The isolate name, country of origin and GenBank accession numbers for the *env* nucleotide sequences for published sequences included in this study are: * Subtype A : Petaluma (USA) M25381, Dixon (USA) L00608, PPR (USA) M36968, USCAhny11 (USA) U02402, USCAlemy08 (USA) U02408, DEBA91 (Germany) AF531043, UK2 (United Kingdom) X69494, UK8 (United Kingdom) X69496, UT113 (Netherlands) X60725, SwissZ2 (Switzerland) X57001, Wo (France) L06135, Sendai1 (Japan) D37813. *Subtype B : TM2 (Japan) M59418, Yokohama (Japan) D37812, Sendai2 (Japan) D37814, Aomori2 (Japan) D37817, Italy M2 (Italy) X69501, ATVIa33 (Austria) AF531045, LP9 (Argentina) D84497. *Subtype C : CABCPady02C (Canada) U02392, CABCPbar07 (Canada) U02397, VND-8 (Vietnam) AB083509, TI-1 (Taiwan) AB016025, MU-1 AB016666 (Taiwan). *Subtype D : Shizuoka (Japan) D37811, Fukuoka (Japan) D37815. *Subtype E : LP-3 (Argentina) D84496, LP-20 (Argentina) D84498, LP-24 (Argentina) D84500.

recorded for 39 cats and 36 of these were domestic cats. Data on clinical status of infected cats were available for most cases. Only two cats in this survey were considered to be clinically 'normal'. Inflammation of the mouth including gingiva and periodontal tissue was a common clinical condition cited. Other reported clinical signs included non-specific signs such as weight loss, anorexia/inappetence, lethargy, pyrexia, skin disease, diarrhoea, as well as kidney disease, liver disease, neurological dysfunction, haematological abnormalities and neoplasia.

Sequence analysis

Nucleotide sequences encompassing up to 695bp of the V3-V5 region of the *env* gene were obtained by PCR from 41 FIV antibody positive cats from four Australian cities (Brisbane, Melbourne, Perth and Sydney). Strong conservation of some amino acid sites was noted. Cysteine residues were completely conserved among the aligned sequences. In addition, *N*-linked glycosylation sites, which occur at the consensus asparagine-X-serine or asparagine-X-threonine,³⁰ were also relatively well conserved among the isolates with only one amino acid change in each of one Australian and three non Australian isolates (data not shown). The conserved nature of these sites is well documented for FIV and suggests an importance to the structure and function of the envelope glycoprotein.^{7, 12, 18}

Phylogenetic analysis

All samples except one Sydney sample (S13) showed a maximum pairwise nucleotide distance of 18.80% with an average distance of 9.02% and a maximum pairwise amino acid distance of 21.33% with an average of 11.16%. The isolates were most similar to subtype A isolates, with nucleotide divergences of 3.44 to 15.98% (amino acid divergences of 2.69 to 21.50%) but larger sequence divergences to the other subtypes (17.99 to 30.84% for nucleotide sequences and 10.53 to 29.09% for amino acid sequences). The Sydney sample S13 showed unusually large pairwise nucleotide divergences of 20.01 to 25.42% (16.29 to 23.04% amino acid divergences) from the other Australian isolates.

The majority of Australian isolates cluster within subtype A on phylogenetic analysis (Figure 1) whereas S13 groups with other subtype B isolates. Hence, subtype A appears to be predominant among Australian cats. The different methods employed to create phylogenetic trees produced trees with similar topologies. No clustering due to geographical location was evident amongst the Australian isolates.

The cell culture maintained T91 isolate does not appear to fall as clearly within the defined subtypes. A GenBank database similarity search was performed with this sequence and it appeared to be almost equally similar to subtype A isolates (eg Petaluma 87%), subtype B isolates (for example Aomori2 88%) and to A/B recombinant isolates (eg CaONAB03 87%). The distance matrix showed 7.27% to 16.67% and 11.42% to 20.28% nucleotide divergences from subtypes A and B isolates respectively and 17.73% to 26.00% nucleotide divergences from the other three subtypes. A similar comparison using the translated T91 sequence showed 8.47% to 17.56% and 8.82% to 14.29% amino acid divergences from subtypes A and B isolates respectively and amino acid divergences from the other three subtypes of 13.56% to 22.90%. Although there was a crossover in distances between subtypes A and B, T91 clearly fell within subtype A on phylogenetic analysis.

Discussion

This study provides the first comprehensive evidence of the prevalent subtype(s) of FIV in Australia. Subtype A was prevalent among the 41 samples analysed from FIV positive cats from four Australian cities. This supports previous studies that found subtype A by heteroduplex mobility analysis of the V3-V4 region of the *env* gene and also sequence analysis of the *gag*, *pol* and LTR regions of a small number of Australian viruses.^{11,14,15} However, we also identified one subtype B virus among the Australian samples. The origin of infection for this cat is unknown.

No clustering of isolates from similar geographical regions was observed in this study, nor did the Australian isolates form a distinct subcluster within subtype A. Geographical clustering has been documented in some countries in which surveys have been conducted, such as Japan.¹² The presence of subclusters within a subtype based on geographical location has been observed with Austrian and Portuguese isolates. Isolates from both countries were found to group together within subtype B.^{10,13} Geographical clustering of isolates has also been documented with HIV.³¹

Genome variability in lentiviruses, including FIV, is largely due to the high error rate of the reverse transcriptase enzyme. However, recombination, particularly in association with super-infection, also plays a role in generating genetic diversity. These phenomena have been demonstrated both *in vitro* and *in vivo* under experimental conditions.^{15,32,33} The occurrence of recombination during natural infection, in areas where more than one subtype exists, is becoming increasingly evident.³⁴ The T91 isolate described in this study may be an example of recombination between subtypes A and B because it is almost equally divergent from subtypes A and B. However, further studies would be required to confirm this isolate as a recombinant.

The evolving nature of lentiviruses means that the emergence of new FIV subtypes is likely to occur. Evidence for this is already apparent in the United States where the V3-V4 sequences of a group of feral cats were found to form a distinct cluster between subtypes B and E.³⁵ To compare with HIV, since its discovery in 1983, HIV-1 strains have been divided into three phylogenetic groups (M – main, O – outliers, N – non-M/non-O) with 24 genetic forms occurring within the main group alone, 13 of which are the result of recombination.³¹ The emergence of different genetic variants may influence the pathogenicity of the virus. In HIV positive individuals, recombinant isolates are thought to have a greater propensity for transmission.³¹

The substantial and ongoing increase in genetic diversity of FIV has also made the development of a successful vaccine difficult. The Fel-O-Vax FIV® dual subtype vaccine, has been shown experimentally to provide good protection against homologous and heterologous subtype challenge.²²⁻²⁴ Its efficacy in the field had been uncertain. However, a recent study which was designed to simulate natural conditions has supported its efficacy against diverse FIV strains.²² It was found to protect against a subtype B (Aomori2) infection when vaccinated, unvaccinated and infected cats were mingled. The experimental data to date suggest that the vaccine would provide protection against Australian FIV isolates.

With the advent of this new vaccine, the development of new FIV diagnostic assays will become important. At present, most existing diagnostic tests rely on the detection of FIV antibodies.

Recently vaccinated cats would also be expected to test positive for FIV antibodies. Therefore, diagnosis may depend on detecting the presence of nucleic acid such as proviral DNA. Knowledge of the prevalent subtypes will aid in the development of assays as sequence variability is likely to influence the sensitivity of these tests. Accumulation of FIV sequence data will allow the development of assays that target more conserved regions of the genome.

In conclusion, this study has found that among FIV in the domestic cat population of Australia, subtype A predominates although subtype B is also present. This knowledge will assist in our understanding of lentiviruses and in the development of therapeutic, preventative and diagnostic strategies.

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